Published in "The Plant Journal 83(5): 818–830, 2015"
which should be cited to refer to this work.

The auxin transporter, OsAUX1, is involved in primary root and root hair elongation and in Cd stress responses in rice (*Oryza sativa* L.)

ChenLiang Yu[1,†], ChenDong Sun[1,†], Chenjia Shen[2,†], Suikang Wang[1], Fang Liu[1], Yan Liu[1], YunLong Chen[1], Chuanyou Li[3], Qian Qian[4], Bibek Aryal[5], Markus Geisler[5], De An Jiang[1,*] and YanHua Qi[1,*]

[1]*State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310058, China,*

[2]*College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China,*

[3]*State Key Laboratory of Plant Genomics, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China,*

[4]*State Key Laboratory of Rice Biology, China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China, and*

[5]*Department of Biology – Plant Biology, University of Fribourg, Rue Albert Gockel 3, CH-1700 Fribourg, Switzerland*

*For correspondence (e-mails dajiang@zju.edu.cn or qyhjp@zju.edu.cn).
†These authors contributed equally to this work.

SUMMARY

Auxin and cadmium (Cd) stress play critical roles during root development. There are only a few reports on the mechanisms by which Cd stress influences auxin homeostasis and affects primary root (PR) and lateral root (LR) development, and almost nothing is known about how auxin and Cd interfere with root hair (RH) development. Here, we characterize rice *osaux1* mutants that have a longer PR and shorter RHs in hydroponic culture, and that are more sensitive to Cd stress compared to wild-type (Dongjin). *OsAUX1* expression in root hair cells is different from that of its paralogous gene, *AtAUX1*, which is expressed in non-hair cells. However, OsAUX1, like AtAUX1, localizes at the plasma membrane and appears to function as an auxin tranporter. Decreased auxin distribution and contents in the *osaux1* mutant result in reduction of *OsCyCB1;1* expression and shortened PRs, LRs and RHs under Cd stress, but may be rescued by treatment with the membrane-permeable auxin 1-naphthalene acetic acid. Treatment with the auxin transport inhibitors 1-naphthoxyacetic acid and *N*-1-naphthylphthalamic acid increased the Cd sensitivity of WT rice. Cd contents in the *osaux1* mutant were not altered, but reactive oxygen species-mediated damage was enhanced, further increasing the sensitivity of the *osaux1* mutant to Cd stress. Taken together, our results indicate that OsAUX1 plays an important role in root development and in responses to Cd stress.

Keywords: auxin, Cd stress, OsAUX1, Primary root, rice (Oryza sativa L.), root hair.

INTRODUCTION

Auxin moves within the plant body through non-polar auxin and polar auxin transport, the latter involving carrier-mediated transport (Kramer and Bennett, 2006). Polar auxin transport plays an important role in various aspects of plant growth and development (Friml and Palme, 2002; Swarup and Bennett, 2003). Transporters of auxin include the influx transporters of the AUXIN1/LIKE AUX1 (AUX1/LAX) family (Bennett *et al.*, 1996; Kerr & Bennett 2007; Swarup *et al.*, 2008), and efflux transporters encoded by members of the PIN-FORMED (PIN) and ATP binding cassette B/P-glycoprotein (ABCB/PGP) families (Noh *et al.*, 2001; Murphy *et al.*, 2002; Geisler *et al.*, 2005; Petrasek *et al.*, 2006; Cho *et al.*, 2007; Yang and Murphy, 2009). In recent years, PIN-LIKE proteins have also been reported to be involved in auxin transport (Barbez *et al.*, 2012). Polar auxin transport in plants creates local auxin maxima that form the basis for plant development and differentiation, regulation of embryogenesis, organogenesis, vascular tissue formation, lateral root initiation and tropic responses (Vieten *et al.*, 2007; Petrasek and Friml, 2009; Peret *et al.*, 2012).

In Arabidopsis, the AUX1/LAX family consists of four highly conserved members, *AUX1*, *LAX1*, *LAX2* and *LAX3*,

which have auxin uptake functions (Peret *et al.*, 2012). AUX1 mediates the transport of indole-3-acetic acid (IAA) in Arabidopsis (Bennett *et al.*, 1996). AUX1, which is asymmetrically localized at the plasma membrane of root protophloem cells, is proposed to promote the acropetal post-phloem movement of auxin to the root apex (Swarup *et al.*, 2001). AUX1 and LAX3 influence lateral root development (Marchant *et al.*, 2002; Swarup *et al.*, 2008), while LAX2 regulates vascular patterning in cotyledons (Peret *et al.*, 2012). In rice, the AUX1/LAX family has five members, which are homologous to *AUX1* of Arabidopsis (Shen *et al.*, 2010). *OsAUX1* that was recently reported to function in the regulation of lateral root development and gravitropism (Zhao *et al.*, 2015), but the function of other OsAUX members is not yet known.

The role of auxin in plant growth and development has been investigated in detail, but only a few studies have examined a possible role of auxin in regulating plant tolerance to abiotic stress, especially that caused by heavy metals. Cd is a major environmental pollutant and causes inhibition of plant growth and alteration of metabolism, leading to a decline in crop productivity. Further, it results in severe human health hazards through food chain contamination (Srivastava *et al.*, 2014). Interestingly, alterations in IAA levels appear to be related to the activity of IAA oxidases in seedlings of pea (*Pisum sativum*) under Cd stress (Chaoui and El-Ferjani, 2005). Further, Cd stress may interfere with the metabolism of auxin, which triggers an increases in the activity of Gretchen Hagen3 (GH3) enzymes in poplar (*Populus* × *canescens*) (Elobeid *et al.*, 2012). Finally, cadmium interferes with the maintenance of auxin homeostasis in Arabidopsis seedlings (Hu *et al.*, 2013). However, the molecular mechanisms underlying the effects of Cd stress on auxin transport and homeostasis in rice are still unclear.

In this study, we report the roles of the auxin transporter, OsAUX1, in regulating primary root (PR) and root hair (RH) development, and in rice tolerance to oxidative stress caused by Cd treatment. We found that three independent *osaux1* loss-of-function mutants were significantly more sensitive to Cd stress than WT. In *osaux1* mutants, Cd increased the levels of the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS) compared with WT, suggesting a role for OsAUX1 in auxin transport during plant tolerance mechanisms to Cd stress via reactive oxygen species-mediated signaling.

RESULTS

Characterization of *OsAUX1* loss- and gain-of-function rice lines

To understand the biological functions of OsAUX1 in rice, three T-DNA insertion mutants of *OsAUX1* [*osaux1-1* (3A-51110), *osaux1-2* (1A-20543) and *osaux1-3* (3A-01770)] were isolated, and over-expression lines of OsAUX1 (*OsAUX1-O1*, *-O2* and *-O3*) were generated (Figure 1). T-DNA insertions were identified in the 3rd exon, 5th intron and 6th exon of the *OsAUX*1 gene by sequencing and comparison with the SIGnAL database at http://signal.salk.edu/cgi-bin/RiceGE (Figure 1a). Homozygous loss-of-function mutants of *OsAUX1* were selected by DNA level and RNA level identifying (Figure 1b,c and Table S1). In the over-expression lines of *OsAUX1*, *OsAUX1* expression was found to be increased eight- to tenfold (Figure 1d).

The PRs of the three *osaux1* lines were all 30% longer than those of the wild-type (WT) (Dongjin, DJ) grown under hydroponic culture conditions for 7 days, while PRs of *OsAUX1* over-expression lines were 30% shorter than those of the WT (Figure 1e). PRs of *osaux1* mutants were insensitive to IAA and the synthetic auxin and herbicide 2,4-dichlorophenyloxy acetic acid (2,4-D), but over-expression lines were hyper-sensitive to them. Interestingly, PRs of the three *osaux1* mutants as well as the *OsAUX1* over-expressing lines were sensitive to 1-naphthalene acetic acid (NAA), which diffuses into cells and thus rescues *osaux1* mutants (Figure 1e).

A phenotypic analysis revealed that the mean lengths of the RHs of *osaux1* were one-third those of WT (Figure 1f and Figure S1). Interestingly, the shorter RHs in *osaux1* mutants were not rescued by IAA and 2,4-D treatments but were not rescued by NAA treatment. These results indicate that mutation of *OsAUX1* disturbs IAA and 2,4-D transport, which depend on auxin transporters, but does not influence NAA influx, which is AUX1/LAX-independent.

OsAUX1 expression in root hair cells and OsAUX1 localization at the cell membrane

In the dicot Arabidopsis, *AUX1* was reported not to be expressed in root hairs but to regulate root hair development (Jones *et al.*, 2009). Based on the experiments described above, loss of OsAUX1 function had an effect on rice RH elongation and thus also showed a defect in RH development. Therefore, we wished to determine whether the molecular mechanisms of AUX1 regulation of RH development are the same in rice and Arabidopsis. The expression pattern of *OsAUX1* was investigated using transgenic rice expressing *ProOsAUX1:OsAUX1-sGFP* and *ProOsAUX1:GUS* (Figure 2 and Figure S1). Using these constructs, *OsAUX1* was constitutively expressed in each tissue (Figure S2) as described by Zhao *et al.* (2015). Expression of *ProOsAUX1:OsAUX1-sGFP* was detected during root hair initiation and when RHs were <250 μm long, but was not found in mature RHs that were more than 800 μm long (Figure 2a). In 3-day-old seedlings, *ProOsAUX1:GUS* staining was observed in all RHs, including those close to the root cap and the proximal zone. However, in 5-day-old seedlings,

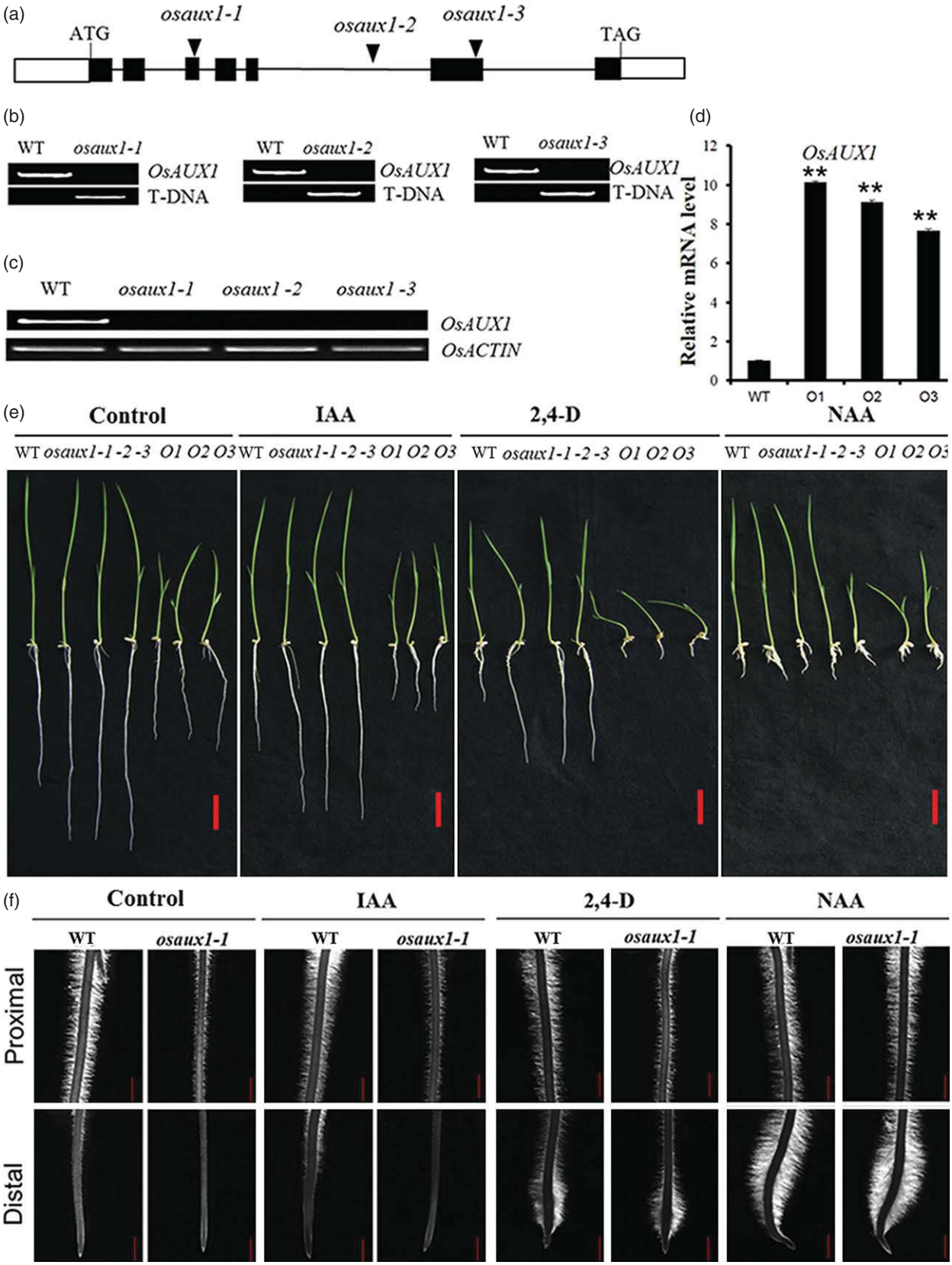

Figure 1. Establishment of *osaux1* and *OsAUX1* over-expression lines.
(a) T-DNA insertion sites in the *osaux1-1*, *osaux1-2* and *osaux1-3* mutants. Black boxes represent exons, white boxes represent untranslated regions, and black lines represent introns. Inverted triangles indicate T-DNA insertion sites.
(b) PCR analysis of T-DNA insertion sites in *osaux1-1*, *osaux1-2* and *osaux1-3*. Upper bands indicate the *OsAUX1* gene fragment, while lower bands indicate T-DNA insertion fragments.
(c) RT-PCR analysis of *OsAUX1* expression. Upper bands indicate the abundance *OsAUX1* (32 cycles), while lower bands show the abundance of the internal control *OsACTIN* (26 cycles).
(d) Quantitative RT-PCR analysis of *OsAUX1* expression. Relative mRNA levels of *OsAUX1* in WT and three *OsAUX1* over-expression lines, *OsAUX1-O1*, *-O2* and *-O3*. *OsACTIN* (Os03g50885) was used as an internal control. Three independent biological repeats were performed. Asterisks indicate statistically significant differences compared with WT (**$P < 0.01$; Student's *t* test)
(e) Phenotypic characterization of WT and *OsAUX1* mutants under control and various auxin treatments. From left to right: seedlings of WT, *osaux1-1 osaux1-2*, *osaux1-3*, *OsAUX1-O1*, *OsAUX1-O2*, *OsAUX1-O3* under control, 1 μM IAA, 0.1 μM 2,4-D and 0.1 μM NAA treatments for 7 days. Scale bars = 2 cm.
(f) Root hair morphology of WT and *osaux1-1* under control, 1 μM IAA, 0.1 μM 2,4-D and 0.1 μM NAA treatments for 3 days. Scale bars = 1 mm.

GUS staining was only detected in the distal zone close to root cap but not in the proximal zone (Figure 2b), i.e. *OsAUX1* was not expressed in mature RHs. These results indicate that *OsAUX1* has a specific spatio-temporal expression in RHs. Moreover, they confirm that *OsAUX1* expression in rice root hair cells is different from that of

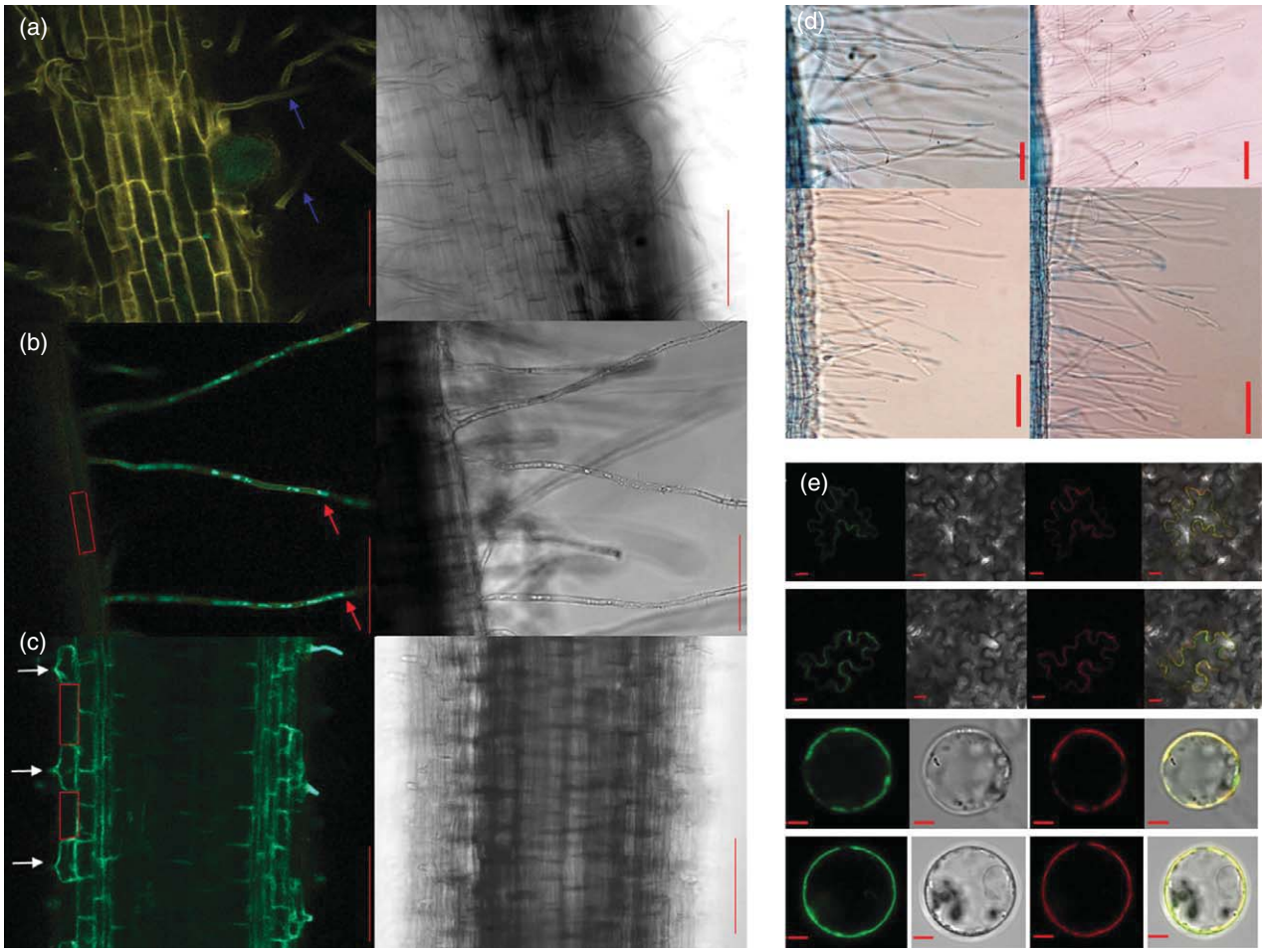

Figure 2. *OsAUX1* expression in RH cells and subcellular localization of OsAUX1.
(a–c) *OsAUX1* expression in developing RH cells. *ProOsAUX1:OsAUX1-sGFP* was transformed into rice WT, and ten positive transgenic lines were imaged by laser scanning fluorescence microscopy. Left, GFP channel (green represents GFP signals; yellow represents auto-fluorescence of rice roots). Right, bright-field images. Scale bar = 100 μm. The images shows *ProOsAUX1:OsAUX1-sGFP* expression in mature RHs (a) (length 800 μm, blue arrows), developing RHs (b) (length 250 μm, red arrows), and cells initiating RHs (c) (white arrows). Red boxes indicate non-root hair cells.
(d) *OsAUX1* expression in RHs of a 3-day-old seedling (left) and a 5-day-old seedling (right). *ProOsAUX1:GUS* was transformed into rice WT, and ten positive transgenic lines were observed. Upper panels indicate the proximal zones of root hairs; lower panels indicate the distal zones of root hairs. Scale bars = 100 μm.
(e) An *OsAUX1–sGFP* fusion construct was transiently expressed in tobacco and rice protoplasts either under the control of the CaMV 35 promoter (1st and 3rd rows) or under the control of its own promoter (2nd and 4th rows). Left to right: green fluorescence of OsAUX1–sGFP, bright-field images, red fluorescence of the protoplast membrane marker pm-rb CD3-1008, and merged microscope images. Scale bars = 10 μm.

its paralogous gene, *AtAUX1*, suggesting functional differences in regulating RH development between monocot and dicot plants.

To better understand the biological function of OsAUX1, we first investigated its subcellular localization. OsAUX1 was localized by transient expression of *35S:OsAUX1-sGFP* and *ProOsAUX1:OsAUX1-sGFP*. Co-localization with the plasma membrane marker pm-rb CD3-1008 (Nelson *et al.*, 2007) in leaf epidermal cells of *Nicotiana benthamiana* and rice protoplasts (Figure 2c) indicates a plasma membrane location as found for AtAUX1, implying that OsAUX1 and AtAUX1 share transport functionality on the plasma membrane.

Cadmium stress induces *OsAUX1* expression in the PRs, LRs and RHs of rice

In recent years, scientists have started paying close attention to the relationship between auxin signaling and stress phenomena caused by heavy metals, such as cadmium (Strader and Bartel, 2009; Zhao *et al.*, 2012; Hu *et al.*, 2013; Tamas *et al.*, 2014). To investigate the *in vivo* function of OsAUX1 in relation to Cd stress, we first tested *OsAUX1* expression under $CdCl_2$ treatment using quantitative RT-PCR and *ProOsAUX1:GUS* promoter analyses. Quantitative RT-PCR showed that *OsAUX1* expression, and that of its homologs *OsAUX2*, *OsAUX3*, *OsAUX4* and *OsAUX5*, increased two- to fivefold by treatment with $CdCl_2$ for 12 h (Figure 3a). Analyses of *ProOsAUX1:GUS* and *ProOsAUX1:OsAUX1:sGFP* rice plants further demonstrated that OsAUX1 expression was distinctly induced in PRs, lateral roots (LRs) and RHs by 50 μM $CdCl_2$ treatment (Figure 3b–e). These results suggest that OsAUX1 functions in Cd stress responses.

osaux1 mutants are sensitive to Cd stress but such sensitivity is alleviated by a low concentration of NAA

To understand the molecular mechanism of OsAUX1 responses to Cd stress, phenotypes of WT and *osaux1* mutants were observed after treatment with Cd (50 μM $CdCl_2$) and/or auxin (10 nM NAA) for 7 days. We found that the length of PRs in WT was inhibited by 20% by $CdCl_2$ treatment, while that in *osaux1* was inhibited by 50% (Figure 4a,c). The number of LRs in WT decreased by 35%, while that in *osaux1-1* decreased by 70% (Figure 4b,c) in response to $CdCl_2$ treatment. The lengths of RHs in *osaux1-1* were much shorter than in WT under $CdCl_2$ treatment (Figure 4b,c) and the RH length of *osaux1-1* was 45% decreased, while those of WT decreased by only 25%. Interestingly, the repression of root growth and development caused by Cd stress was retarded by simultaneous NAA application as indicated by PR length and LR density. Further, RH growth in the *osaux1-1* mutant under Cd+NAA treatment was significantly increased compared to Cd treatment alone (Figure 4). Root growth responses to Cd+IAA treatment show similar trends to Cd+NAA treatment (Figure S3). These results imply that the sensitivity to Cd stress may be dependent on local changes in auxin concentrations caused by reduced auxin transport in *osaux1-1*.

osaux1-1 shows reduced auxin contents and distribution under control and IAA treatments and under Cd stress

To confirm that altered auxin contents do indeed have an effect on the sensitivity to Cd stress in rice roots, the auxin reporter DR5-GUS was transformed into WT and *osaux1-1*. IAA induced DR5 expression in WT and *osaux1-1*, while

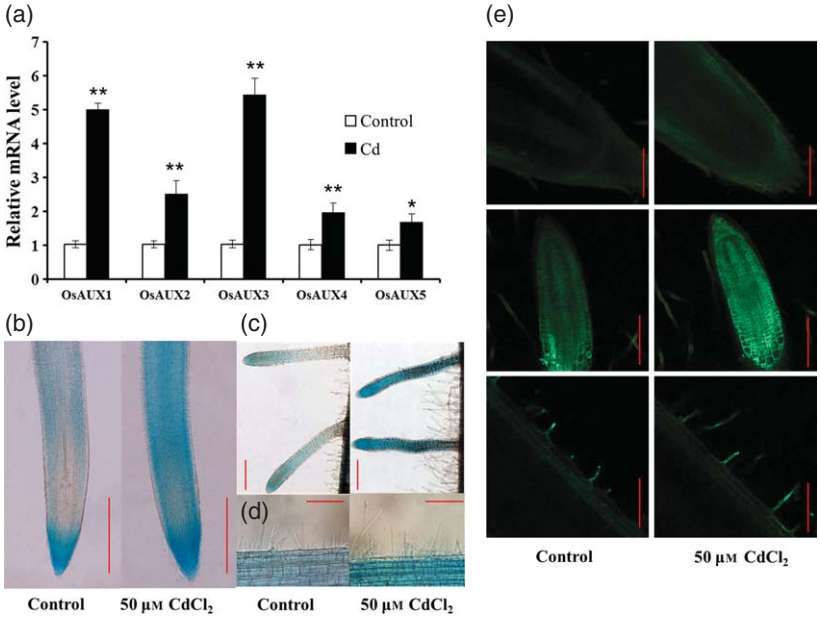

Figure 3. Expression pattern of *OsAUX1* in roots under Cd treatment.
(a) Quantitative RT-PCR analysis of *OsAUX* gene expression in 5-day-old roots under control and 50 μM $CdCl_2$ treatments for 12 h. Three independent biological repeats were performed. Asterisks indicate statistically significant differences compared with control (*$P < 0.05$, **$P < 0.01$; Student's *t* test).
(b–d) Expression pattern of *ProOsAUX1:GUS* in PRs (b), LRs (c) and RHs (d) of 5-day-old seedlings under hydroponic culture (control, left) and 50 μM $CdCl_2$ treatment (right) for 12 h. Scale bars = 500 μm. Ten seedlings were tested in this experiment.
(e) Expression pattern of *ProOsAUX1:OsAUX1:sGFP* in PRs (top), LRs (middle) and RHs (bottom) of 5-day-old seedlings under hydroponic culture (control, left) and 50 μM $CdCl_2$ treatment (right) for 12 h. Scale bars = 100 μm.

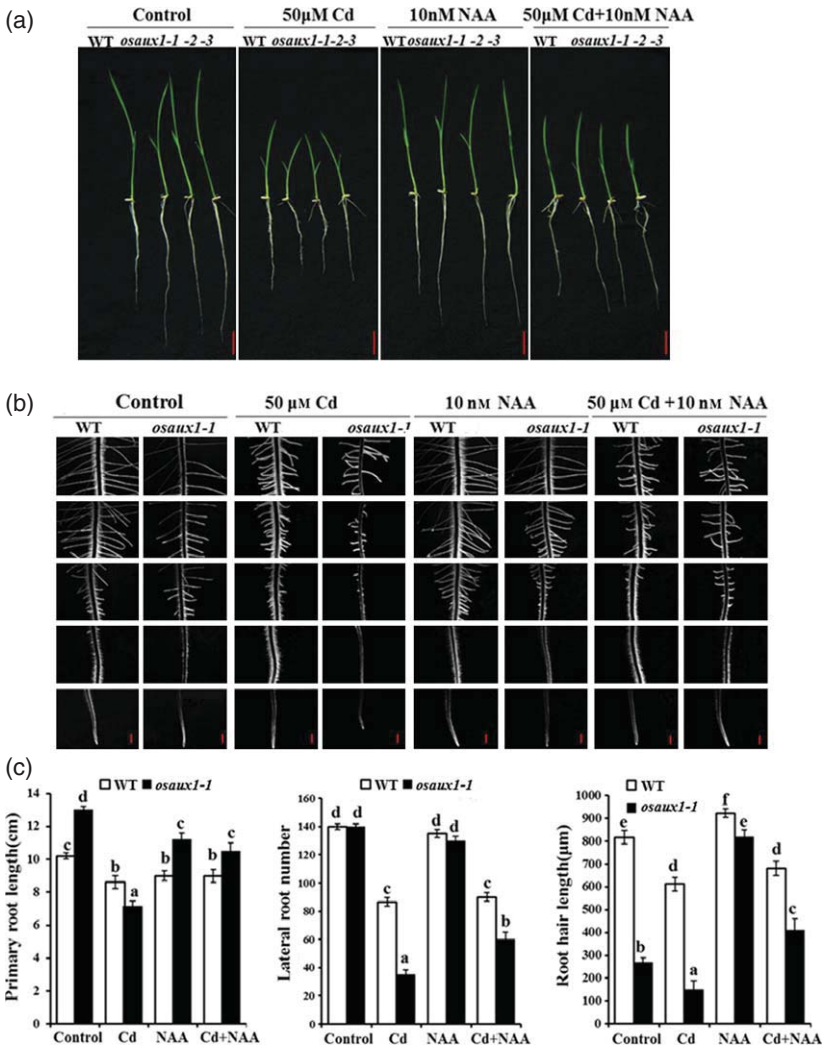

Figure 4. Effect of Cd stress on WT and *osaux1* roots.

(a) Phenotype of WT and *osaux1* roots exposed to Cd stress. Left to right: WT, *osaux1-1*, *osaux1-2* and *osaux1-3* grown under control conditions or treated with 50 μM $CdCl_2$, 10 nM NAA or 10 nM NAA plus 50 μM $CdCl_2$ for 7 days. Scale bars = 2 cm.

(b) Phenotype of LRs and RHs in WT and *osaux1* under Cd stress. Left to right: WT and *osaux1-1* mutant grown on under control conditions or treated with 50 μM $CdCl_2$, 10 nM NAA or 10 nM NAA plus 50 μM $CdCl_2$ for 7 days. Scale bars = 1 mm.

(c) Quantification of root phenotypes. PR lengths, LR numbers and RH lengths were measured in WT and the *osaux1-1* mutant grown under control conditions or treated with 50 μM $CdCl_2$, 10 nM NAA or 10 nM NAA plus 50 μM $CdCl_2$ for 7 days. Ten seedlings were assessed for each treatment. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).

$CdCl_2$ reduced it compared to controls (Figure 5a). However, DR5-GUS activity in *osaux1-1* was always lower than in WT under control, IAA and Cd treatments (Figure 5a,b). In agreement, auxin contents in *osaux1-1* were also reduced compared to WT under the conditions described above (Figure 5c).

In order to investigate whether reduced auxin contents were caused by defects in polar auxin transport, we examined acropetal and basipetal transport of [^{3}H]-IAA under control and Cd stress conditions (Figure 5d). Acropetal transport of [^{3}H]-IAA in *osaux1-1* was significantly lower than in WT both under control and Cd stress conditions. In contrast, basipetal transport of [^{3}H]-IAA in *osaux1-1* was higher than in WT under control conditions, but was strongly decreased under Cd stress. These results coincide with phenotypes for *osaux1-1* under Cd stress, and suggest that reduced polar auxin transport in *osaux1-1* is the primary cause for decreased auxin contents. Our data also indicate that Cd treatments may negatively regulate polar auxin transport.

On the other hand, when 7-day-old WT seedlings were treated with 50 μM $CdCl_2$ for 5 days, the PR length was 15% shorter than for plants treated simultaneously with the auxin transport inhibitors 1-naphthoxyacetic acid (NOA) and *N*-1-naphthylphthalamic acid (NPA) (Figure 5e), indicating that both transport inhibitors significantly reduced the tolerance of WT rice plants to Cd stress. Taken together, these results indicate that decreased auxin contents and distribution are responsible for increased sensitivity toward Cd stress, suggesting that auxin plays an important role as a signaling molecular in response to Cd stress.

OsCyCB1;1 expression in *osaux1-1* is decreased under control and IAA treatments and Cd stress

Cell-cycle regulation plays a crucial role in crop plants during Cd stress, as exemplified by the finding that cell cycle-related genes in soybean are induced by Cd treatment (Swarup and Bennett, 2003). To study further how OsAUX1 affects Cd stress, i.e. the relationship among

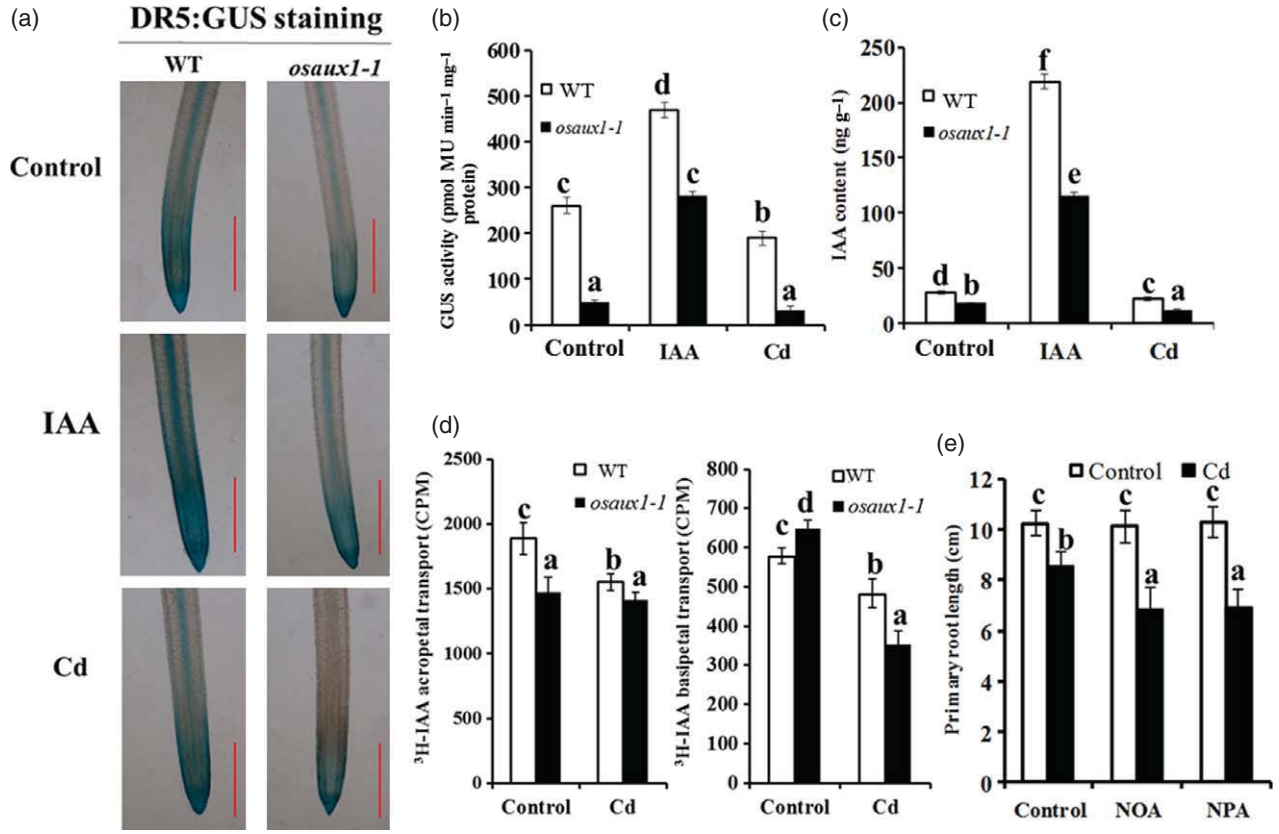

Figure 5. Auxin levels of WT and the *osaux1-1* mutant under IAA/Cd stress, and the WT response to Cd stress after application of NOA or NPA.
(a) GUS staining in primary roots in 5-day-old *ProDR5: GUS* transgenic WT and *osaux1-1* seedlings treated with control, 1 μM IAA or 50 μM $CdCl_2$ for 5 days. Scale bars = 100 μm.
(b) *ProDR5:GUS* activity in the lines shown in (a). Ten seedlings were assessed under each condition. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).
(c) Measurement of auxin concentrations. The roots of WT and *osaux1-1* mutants were treated with control, 1 μM IAA or 50 μM $CdCl_2$ for 5 days, and then their auxin concentrations were measured using GC-MS. Five biological repeats were used for each condition; letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).
(d) Analysis of acropetal and basipetal ^{3}H-IAA transport. WT and *osaux1-1* roots were treated with solvent or 50 μM $CdCl_2$ for 5 days. Left panel, acropetal transport of ^{3}H-IAA; right panel, basipetal transport of ^{3}H-IAA. Twenty independent biological repeats were analyzed for each experiment; letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).
(e) Effect of auxin transport inhibitors on responses to Cd stress. WT seeds were pre-treated with either nutrient solution (control), 10 μM NOA or 0.1 μM NPA for 2 days before transfer to nutrient solution containing 50 μM $CdCl_2$. PR lengths were measured at 5 days after $CdCl_2$ treatment; ten biological repeats were performed for each experiment. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).

auxin, cell-cycle regulation and Cd stress, we transferred the reporter *ProCyCB1;1:GUS* into WT and *osaux1-1*. *ProCyCB1;1:GUS* staining in WT and *osaux1-1* was increased by IAA, NAA and Cd treatments compared to control conditions (Figure 6a). However, the staining intensity in *osaux1-1* under the above conditions was significantly lower than in WT, suggesting that this is caused by decreased auxin contents in *osaux1-1* (Figure 6b). Quantitative RT-PCR analysis of *OsCyCB1;1* expression verified the reporter gene data (Figure 6c). These results suggest that OsAUX1 affects the ability of rice plants to respond to Cd stress via OsCyCB1;1. Reduced *OsCyCB1;1* expression in *osaux1-1* appears to further decrease cell division, and leads to shorter PRs compared to WT.

Cd contents are not altered in *osaux1*

P_{1B}-type heavy-metal ATPases (HMAs) are transmembrane metal-transporting proteins that play a key role in metal homeostasis (Lee *et al.*, 2007). OsHMA3 in yeast transports Cd into the ER and thus increases Cd sensitivity (Ueno *et al.*, 2010). Expression of three *OsHMA* genes, *OsHMA5*, *OsHMA6* and *OsHMA9*, was induced by Cd stress, and these were therefore suggested to play roles in heavy-metal detoxification (Lee *et al.*, 2007). We tested the expression of the above genes in WT and *osaux1-1* under Cd treatment for the first 3 days after germination. All four genes (but especially *OsHMA3*) were significantly up-regulated in *osaux1-1* compared to the WT under Cd stress

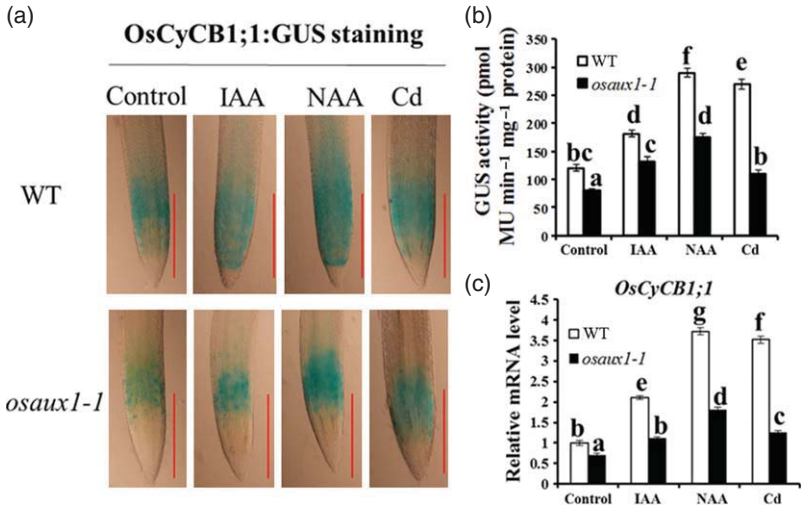

Figure 6. Characterization of *OsCyCB1;1* expression in WT and *osaux1-1* under IAA and Cd treatments.
(a) GUS staining in primary roots. Five-day-old *ProOsCyCB1;1:GUS*-transformed WT and *osaux1-1* seedlings were treated with the solvent (control), 1 μM IAA, 10 nM NAA or 50 μM $CdCl_2$ for 5 days. Scale bars = 500 μm.
(b) GUS activity in primary roots of the above material. Ten seedlings were assessed under each condition. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).
(c) Quantitative RT-PCR analysis of the expression of *OsCyCB1;1* in WT and *osaux1-1* under control, IAA, NAA or $CdCl_2$ treatment for 5 days. *OsACTIN* was used as an internal control. Quantitative RT-PCR experiments comprised three independent biological repeats. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).

(Figure 7a). In a previous report, *OsHMA3* expression in yeast provided increased Cd sensitivity (Ueno *et al.*, 2010). Therefore, Cd contents in WT and *osaux1-1* roots were quantified by inductively coupled plasma optical emission spectrometry. However, no differences in Cd contents between WT and *osaux1-1* roots were detected (Figure 7b). Quantification of Cd contents in WT and *osaux1-1* PRs and LRs using the Cd dye Leadmium™ Green AM also revealed no significant difference (Figure 7c). These results imply that OsAUX1 does not affect rice Cd sensitivity by altering Cd contents. However, the underlying mechanisms need to be further explored.

Cd stress induces the production of reactive oxygen species, which trigger cell death in plants (Sabater and Martin, 2013; Dubey *et al.*, 2014). To understand the increased sensitivity of *osaux1* to Cd stress, we quantified $O_2^{\cdot-}$, H_2O_2 and TBARS and assessed cell death (Figure 7d). The amount of $O_2^{\cdot-}$ produced by the mitochondrial electron transport chain was increased in *osaux1-1* compared to the WT under Cd treatment. H_2O_2 has been reported to be closely related to root growth in rice under Cd stress (Zhao *et al.*, 2012). We quantified H_2O_2 from control and Cd-treated 1–5-day-old seedlings. H_2O_2 concentrations in *osaux1* were slightly higher than in WT under control conditions, but were significantly less increased in *osaux1-1* under Cd treatment. This suggests a regulating role for auxin transport through OsAUX1 during tolerance to Cd stress in rice via reactive oxygen species-mediated signaling. TBARS was used as an indicator to estimate peroxidation of lipids in the membrane. Increased TBARS levels were observed in the *osaux1-1* mutant under Cd treatment in the first 5 days compared with WT. The cell integrity of the rice root tips was quantitatively analyzed by Evans blue staining. Evans blue accumulated at higher levels in *osaux1-1* under Cd treatment compared to the WT, suggesting that the toxicity and cell death in the *osaux1-1* mutant most likely caused by Cd stress were more severe than in WT. These results indicate that *osaux1* is probably more sensitive to Cd stress due to increased production of reactive oxygen species, which triggers the peroxidation of lipids in membrane, finally causing cell death.

DISCUSSION

Auxin signaling has been reported to be involved in activation of Cd-induced morphogenic defense responses in root tips of barley (*Hordeum vulgare*) (Tamas *et al.*, 2014). Auxin also alleviates Cd toxicity in wheat (*Triticum aestivum*) and Arabidopsis by enhancing antioxidant defense activities (Agami and Mohamed, 2013; Zhu *et al.*, 2013). Auxin transport pathways may therefore be required for Cd-modulated LR development in Arabidopsis (Hu *et al.*, 2013). However, the relationship between auxin transport and responses to Cd stress in rice is less well investigated. Here, we reveal that the auxin transporter OsAUX1 is implicated in responses to Cd stress.

OsAUX1 negatively regulates PR length but positively regulates RH development

In Arabidopsis, AUX1 functions in regulating LR and RH development (Swarup *et al.*, 2008; Jones *et al.*, 2009). In a recent paper, OsAUX1 was reported to function in regulating LR development as in Table S2 (Zhao *et al.*, 2015). Here we show that three independent *osaux1* mutants produce elongated primary roots under normal growth conditions and shorter RHs compared to WT (Figure 1e,f), indicating that OsAUX1 is also involved in PR and RH development. Loss of OsAUX1 function led to increases in PR length, suggesting that OsAUX1 negatively regulates PR length but positively regulates RH development. OsAUX1 is critical for RH development because the shorter RHs in *osaux1* mutants were not rescued by treatment with IAA and 2,4-D, whose influx requires auxin importers, but were rescued

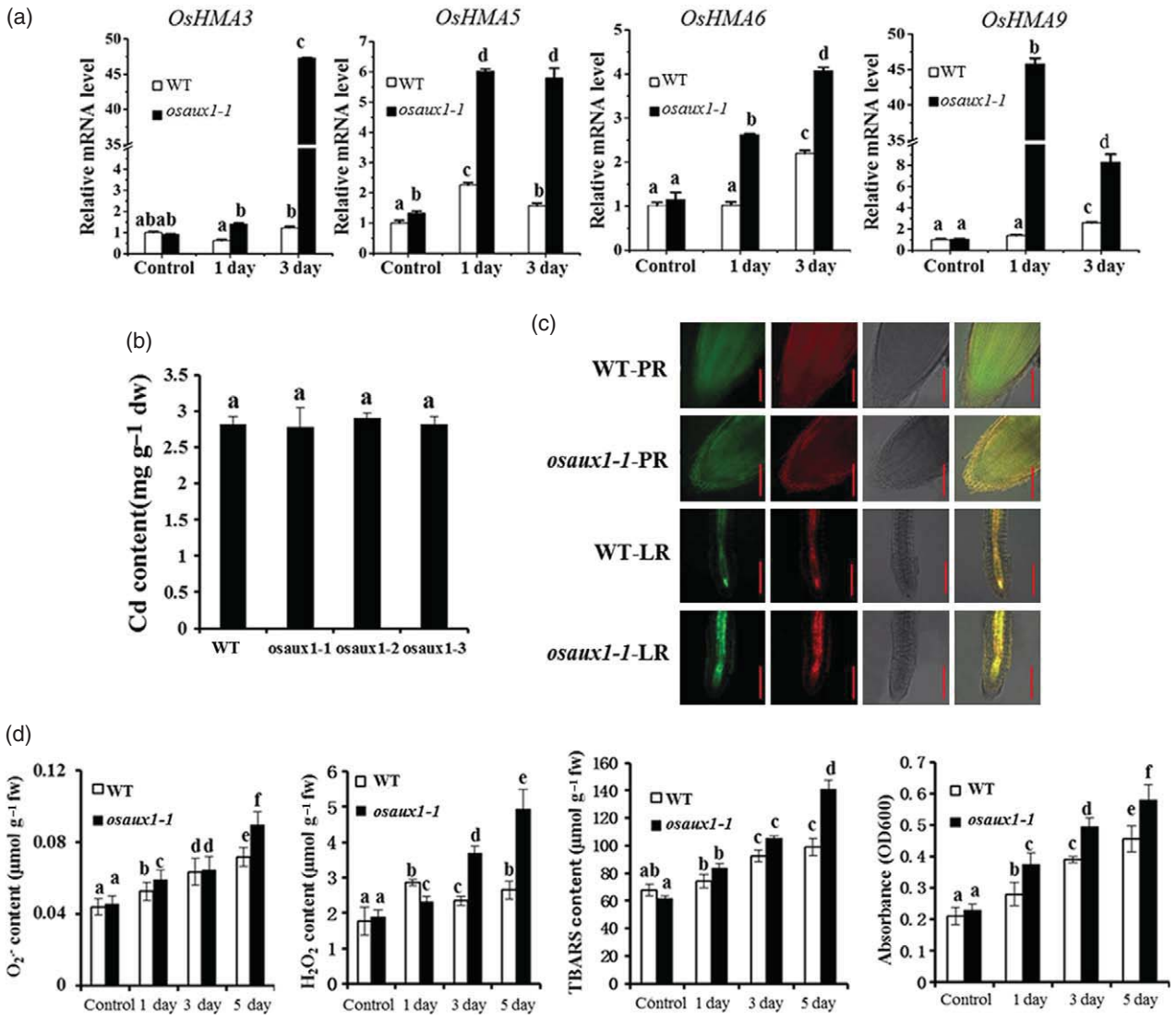

Figure 7. Expression of *OsHMA* genes, localization of Cd and accumulation of Cd in roots of Cd-treated rice plants.
(a) Relative mRNA levels of *OsHMA*3, *OsHMA*5, *OsHMA*6 and *OsHMA*9 in roots of WT and *osaux1-1* after treatment with 50 μM $CdCl_2$ for 0, 1 or 3 days. *OsACTIN* was used as an internal control. Quantitative RT-PCR experiments comprised three independent biological repeats. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).
(b) Root Cd contents of WT, *osaux1-1*, *-2* and *-3*. Rice plants were treated with 50 μM $CdCl_2$ for 5 days. Five biological repeats were used.
(c) Confocal microscopy imaging of Cd in primary roots and lateral roots of WT and *osaux1-1* after 50 μM $CdCl_2$ treatment for 5 days. From left to right: staining using the Cd-specific probe Leadmium™ Green AM, propidium iodide staining, bright-field images and merged images. Scale bars = 100 μm.
(d) Effect of Cd stress on H_2O_2 and $O_2^{\cdot-}$, lipid peroxidation (TBARS) and cell death. Accumulation of $O_2^{\cdot-}$, H_2O_2 and TBARS was measured in WT and *osaux1-1* rice exposed to 50 μM $CdCl_2$ for 0, 1, 3 and 5 days. Cell death was also assessed for of WT and *osaux1-1* exposed to 50 μM $CdCl_2$ for 0, 1, 3 and 5 days. Samples were analyzed by Evans blue staining. Letters indicate significant differences between means, determined using Duncan's multiple range mean comparisons (5% α).

by treatment with NAA, which may enters the cell by free diffusion, thus bypassing importers.

OsAUX1 regulates RH development differently from AUX1 in Arabidopsis

The root epidermis of Arabidopsis includes two cell types: those that form root hairs and those that do not. In Arabidopsis, AUX1 was localized in non-hair epidermal cells, suggesting that auxin transport through non-hair cells sustains root hair development (Jones *et al.*, 2009). *AUX1* expression in non-hair cells demonstrates that AUX1 affects the auxin content of both hair and non-hair cells. However, in rice, OsAUX1 was detected in root hair cells but not non-hair cells (Figure 2a), indicating that OsAUX1-mediated RH development is not the same as in Arabidopsis. Arabidopsis has a type 3 striped pattern of root hairs (Kwak and Schiefelbein, 2007; Horn *et al.*, 2009), while rice may show the type 2 pattern, which depends on

asymmetrical cell divisions (Clowes, 2000). Our data on OsAUX1 localization further support the idea that rice RHs belong to type 2.

AUX1 functions as an auxin influx carrier that is thought to transport charged IAA into the cytoplasm in Arabidopsis (Dharmasiri *et al.*, 2006; Carrier *et al.*, 2008; Peret *et al.*, 2012; Robert and Friml, 2009). OsAUX1 in rice is localized to plasma membranes (Figure 2c), as for AUX1 in Arabidopsis. However, whether OsAUX1 functions as an importer or exporter remains to be confirmed.

Reduced auxin content increases the sensitivity to Cd stress in an action involving OsCyCB1;1

In recent years, evidence has accumulated showing that auxin and Cd stress responses are interconnected. Our data show that *OsAUX1* expression in rice roots is induced by Cd stress (Figure 3), indicating that the *in vivo* function of OsAUX1 may be related to Cd stress responses. A detailed study of the three *osaux1* mutants revealed that their PRs, LRs and RHs are more sensitive to Cd compared to WT, but that the inhibititory effect of Cd was alleviated by a low concentration of NAA (Figure 4). Exogenous NAA treatment improves Cd tolerance in *osaux1* mutants, suggesting that local auxin gradients provided by OsAUX1 are essential for Cd tolerance in rice. Auxin content and distribution were reduced in *osaux1-1* under control, IAA treatment and Cd stress, while application of the auxin transport inhibitors NOA and NPA increased the Cd sensitivity in WT (Figure 5). Taken together, this series of molecular, morphological and biochemical analyses strongly demonstrates that a reduction of auxin content in rice plants increases their sensitivity to Cd stress.

Previous reports had already suggested that auxin signaling and cell-cycle regulation function to regulate plant root development in Arabidopsis, especially under unfavorable conditions (De Smet *et al.*, 2010; Potters *et al.*, 2007; Perez Torres *et al.*, 2009). Our study shows that *OsAUX1* also affects *OsCyCB1;1* expression under IAA and Cd treatments (Figure 6), suggesting that *OsAUX1* and *OsCyCB1;1* may play a role during auxin responses, cell-cycle regulation and Cd stress. However, the genes related to cell-cycle regulation include a large family of 44 cyclins, which are known to be functionally redundant and conserved in rice (Guo *et al.*, 2007). Hence, whether other cell-cycle genes are involved in OsAUX1-mediated responses to Cd stress needs to be addressed in further research.

Increased oxidative damage in *osaux1* results in increased Cd sensitivity

Cd, which usually enters humans through the food chain via plants or cigarette smoke, is highly toxic to most organisms. Hence, inhibiting Cd transport or fixing Cd within the plant root may be a useful strategy for decreasing the accumulation of Cd in humans (Ueno *et al.*, 2010). For example, OsHMA3 controls Cd over-accumulation in rice through transport of Cd into vacuoles, partially limiting the root-to-shoot Cd translocation in rice plants (Miyadate *et al.*, 2011; Tezuka *et al.*, 2010). On the other hand, Cd stress disturbs auxin homeostasis by affecting its distribution and metabolism in Arabidopsis (Hu *et al.*, 2013). In our study, we show that *OsHMA3* and its homologs are significantly up-regulated in *osaux1-1* under Cd stress compared to WT (Figure 7d), suggesting that OsAUX1 may negatively regulate expression of *OsHMA3* and it homologs, explaining why *osaux1-1* is more sensitive to Cd stress. However, we did not detect a difference in Cd content within roots of WT and *osaux1-1*, indicating that the regulatory mechanism mediated by OsAUX1 in response to Cd stress is intricate.

The accumulation of reactive oxygen species under Cd stress is a common phenomenon, which causes oxidative damage in plants. $O_2^{\cdot -}$ is the initial reactive oxygen species produced in response to environmental stresses, such as toxic heavy metals, triggering formation of more reactive oxygen species such as $OH^{\cdot}$ and $^1O^2$ (Halliwell, 2006). H_2O_2 is usually employed as an oxidative stress biomarker and is an important signaling molecule, depending on its cellular concentration. A certain concentration of H_2O_2 appears to be necessary to activate H_2O_2-dependent signaling pathways, stimulating the expression of H_2O_2-responsive genes (Michelet *et al.*, 2013). However, the link between H_2O_2 and the auxin signaling pathway in Cd-stressed rice roots is poorly understood. It has been reported that H_2O_2 may act as a signal influencing auxin signaling and regulating growth of the root system in plants under Cd stress (Zhao *et al.*, 2012). Upon Cd exposure for 1 day, the H_2O_2 concentration was highly increased in WT, and similar results were found in *aux1* of Arabidopsis under arsenite treatment (Krishnamurthy and Rathinasabapathi, 2013). Over-production of $O_2^{\cdot -}$ and H_2O_2 is the cause of oxidative stress, and the concentration of $O_2^{\cdot -}$ and H_2O_2 was higher in *osaux1* than in WT under Cd treatment. Cd-induced lipid peroxidation and damage to membrane stability are well-known consequences of $O_2^{\cdot -}$ and H_2O_2 generation. Our results indicated that lipid peroxidation and cell integrity were not altered differently in *osaux1* compared to the WT under control conditions. However, *osaux1-1* revealed a high degree of oxidative damage under Cd treatment for 5 days, implying that OsAUX1 has a positive impact during the response to Cd stress.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Seeds of WT/Dongjin, *osaux1-1*, *osaux1-2* and *osaux1-3*, and *OsAUX1* over-expression lines were germinated and planted in rice nutrient solution as previously described (Wang *et al.*, 2014;

Xu *et al.*, 2014). Phytohormone treatment was performed using 1 μM IAA, 0.1 μM 2,4-D or 0.1 μM NAA; the duration of treatments is indicated in the figure legends.

Identification of *osaux*1 mutants

T-DNA insertion sites in three independent mutants of *OsAUX1* [*osaux1-1* (3A-51110), *osaux1-2* (1A-20543) and *osaux1-3* (3A-01770)], were determined by comparison with the SIGnAL database at http://signal.salk.edu/cgi-bin/RiceGE. Insertions were confirmed by PCR using Os*AUX1*-specific and T-DNA border primers (2715LB or Ngus-RB, see Figure 1b and Table S3). *OsAUX1* expression in the three *osaux1* mutants was analyzed by RT-PCR using the primers shown in Table S3.

RNA extraction and quantitative RT-PCR

Total RNA was isolated using a plant RNA extract kit according to the manufacturer's instructions (Tiangen, http://www.tiangen.com/). Reverse transcriptase-PCR was performed as described previously (Wang *et al.*, 2010, 2014). The primers used are listed in Table S3.

Construction and transformation of binary vectors

For the *35S:OsAUX1* construct, the ORF of *OsAUX1* was amplified from cDNA of Dongjin (DJ) using the primers listed in Table S3, and then cloned into the binary vector pCAMBIA1300 (http://www.cambia.org/). For the *35S:OsAUX1:GFP* construct, the ORF of *OsAUX1* (without terminator) was cloned into binary vector pCAMBIA1300 containing a *35S:sGFP* cassette. For the *ProOsAUX1:OsAUX1-sGFP* construct, the CaMV 35S promoter was replaced by the native *OsAUX1* promoter (*ProOsAUX1*). For the *ProOsAUX1:GUS* construct, 2 kb upstream of the ATG of *OsAUX1* gene were amplified from genomic DNA of WT, and cloned into binary vector pBI101.3 (Kang *et al.*, 2013). All vectors were introduced into *Agrobacterium* strain EHA105 and transformed into WT rice as described previously (Hiei *et al.*, 1994).

Subcellular localization of OsAUX1

35S:OsAUX1:sGFP and *ProOsAUX1:OsAUX1:sGFP* fusion constructs were transiently expressed in tobacco epidermal cells by *Agrobacterium*-mediated transformation as previously described (Qi *et al.*, 2012). The two constructs were also polyethylene glycol/calcium-transfected into rice protoplasts prepared from stems of 10-day-old rice seedlings (Yoo *et al.*, 2007). Images were acquired using an LSM710 microscope (Zeiss, http://www.zeiss.com/corporate/en_de/home.html).

GUS staining and analysis of GUS activity

GUS staining of WT and *osaux1* seedlings transformed with *ProOsAUX1:GUS*, *DR5:GUS* (Ulmasov *et al.*, 1997) and *ProOsCyCB1;1:GUS* was performed as described previously (Jefferson *et al.*, 1987) After staining, the tissues were soaked in 70% ethanol to remove chlorophyll and surface dyes. Quantification of GUS activity was performed as described by Jefferson *et al.* (1987) by monitoring cleavage of the GUS substrate 4-methylumbelliferyl-β-D-glucuronide (Sigma, http://www.sigmaaldrich.com/china-mainland.html). GUS-stained roots were imaged using a Nikon 16 Eclipse 80i microscope (Nikon Corporation, http://www.nikon-instruments.com.cn/index.html).

Analysis of IAA concentrations and polar auxin transport

For measurement of IAA concentrations, 20 mg of roots of 7-day-old WT and the *osaux1-1* mutant were washed several times with deionized water, and samples were ground into a fine powder under liquid nitrogen. The extraction of IAA was performed as described by Wang *et al.* (2014). IAA purification and quantification were performed as described by Ljung *et al.* (2005) using a FOCUS GC-DSQII gas chromatograph (Thermo Fisher Scientific, http://www.thermofisher.com/en/home.html). Analyses of polar ^{3}H-IAA transport in rice roots (1 cm segments from the tip) was performed using a 1450 MicroBeta TriLux liquid scintillation counter (Perkin-Elmer, http://www.perkinelmer.com/) as described previously (Qi *et al.*, 2008).

Cd localization and quantification

The distribution of Cd in WT and *osaux1-1* after 50 μM $CdCl_2$ treatment for 5 days was visualized using the Cd-specific probe Leadmium™ Green AM (Invitrogen, http://www.lifetechnologies.com/cn/zh/home/brands/invitrogen.html) according to the manufacturer's instructions. Twenty PRs of WT or *osaux1-1* were immersed in the dye solution at room temperature for 3 h in the dark, and then washed with 0.85% NaCl. Roots were stained with propidium iodide for 10 min, washed with 0.85% NaCl for three times at room temperature, and micrographs were obtained by confocal microscopy.

For measurement of Cd concentrations, harvested roots (after treatment with or without 50 μM $CdCl_2$ treatment for 5 days) were washed first in distilled water and then in 0.01 mM EDTA solution, and dried at 80°C until the materials reached constant weights. Then 0.1 g of each sample was digested with HNO_3/H_2O_2 at 110°C for 0.5 h using a microwave digestor (Anton Paar, http://www.anton-paar.com/cn-cn/). The samples were then dissolved in deionized water to 25 ml of constant volume for analysis of Cd content using an OPTIMA 8000DV inductively coupled plasma optical emission spectrometer (Perkin Elmer). Five biological replicates were performed for each sample in all experiments.

Quantification of H_2O_2, $O_2^{\cdot-}$, TBARS and cell death detection

Root H_2O_2 levels were measured as described by Jana and Choudhuri (1981). Root samples (0.1 g) were homogenized in 6 ml phosphate buffer (50 mmol/l, pH 6.5). The absorbance at 410 nm was read using a spectrophotometer (UV-2550, Shimadzu, http://www.shimadzu.com.cn/). Superoxide anion radicals were measured by the hydroxylamine oxidation method (Elstner and Heupel, 1976). The absorbance was measured at 520 nm using the above-mentioned spectrophotometer. Lipid peroxidation was quantified on the basis of TBARS, which represent malondialdehyde and other end products of lipid peroxidation, as described by Hodges *et al.* (1999). The distal 2 cm of the root tips were excised from 40 roots and stained in 0.025% w/v Evans blue solution for 20 min at room temperature, and then washed with double distilled H_2O to remove excessive dye. Root tips were treated with 50% methanol and 1% SDS for 1 h at 50°C, and the absorbance at 600 nm was quantified as described by Baker and Mock (1994).

ACKNOWLEDGEMENTS

We are grateful to Gynheung An (Plant Functional Genomics Laboratory, Yongin, Korea) for providing T-DNA insertional mutants of *OsAUX1*, Akio Miyao (Rice Genome Resource Center, Tsukuba, Japan) for providing the full-length cDNA clones of *OsAUX1*, YongHong Wang and ShuJia Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) for polar auxin transport assays, and Laurence Charrier for technical assistance. This project

was funded by grants from the National Basic Research Program of China (973 Program, grant number 2015CB942900), the National Natural Science Foundation of China (grant numbers 31271692, 31371591 and 31471460), the National Science and Technology Support Plan (2012BAC09B01), the Natural Science Foundation for Distinguished Young Scholars of Zhejiang province, China (LR13C130002), the Program for Innovative Research Team in Universities (IRT1185), the Chinese Fundamental Research Funds for the Central Universities, and the Swiss National Funds (to M.G.).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Root hair length in WT and *osaux1-1* under various treatments.

Figure S2. Expression pattern of *OsAUX1*.

Figure S3. Phenotype of WT and *osaux1* under Cd and IAA co-treatment.

Table S1. PR lengths of 7-day-old seedlings of WT, *osaux1* mutants and *OsAUX1* over-expression lines under various treatments.

Table S2. LR number of 7-day-old seedling of WT, *osaux1* mutants and *OsAUX1* over-expression lines under various treatments.

Table S3. Primers used in this study.

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